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### How to cite this manuscript

If you make reference to this version of the manuscript, use the following information:

Li, C., Chen, M., Chao, S., Yu, J., & Bai, G. (2013). Identification of a novel gene, H34, in wheat using recombinant inbred lines and single nucleotide polymorphism markers. Retrieved from <http://krex.ksu.edu>

### Published Version Information

**Citation:** Li, C., Chen, M., Chao, S., Yu, J., & Bai, G. (2013). Identification of a novel gene, H34, in wheat using recombinant inbred lines and single nucleotide polymorphism markers. *Theoretical and Applied Genetics*, 126(8), 2065-2071.

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**Digital Object Identifier (DOI):** doi:10.1007/s00122-013-2118-5

**Publisher's Link:** <http://link.springer.com/article/10.1007/s00122-013-2118-5>

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**Identification of a novel gene, *H34*, in wheat using recombinant inbred lines and single nucleotide polymorphism markers**

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**Abstract** Hessian fly (HF), *Mayetiola destructor*, is an important pest of wheat (*Triticum aestivum* L.) worldwide. Because it has multiple biotypes that are virulent to different wheat HF resistance genes, pyramiding multiple resistance genes in a cultivar can improve resistance durability, and finding DNA markers tightly linked to these genes is essential to this process. This study identified quantitative trait loci (QTLs) for Hessian fly resistance (HFR) in the wheat cultivar ‘Clark’ and tightly linked DNA markers for the QTLs. A linkage map was constructed with single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers using a population of recombinant inbred lines (RILs) derived from the cross ‘Ning7840’ x ‘Clark’ by single-seed descent. Two QTLs associated with resistance to fly biotype *GP* were identified on chromosomes 6B and 1A, with the resistance alleles contributed from ‘Clark’. The QTL on 6B flanked by loci *Xsnp921* and *Xsnp2745* explained about 37.2% of the phenotypic variation, and the QTL on 1A was flanked by *Xgwm33* and *Xsnp5150* and accounted for 13.3% of phenotypic variation for HFR. The QTL on 6B has not been reported before and represents a novel wheat gene with resistance to HF, thus, it is designated *H34*. A significant positive epistasis was detected between the two QTLs that accounted for about 9.5% of the mean phenotypic variation and increased HFR by 0.16. Our results indicated that different QTLs may contribute different degrees of resistance in a cultivar and that epistasis may play an important role in HFR.

**Keywords** *Triticum aestivum* · Hessian fly · Resistance gene · Quantitative trait loci · Single nucleotide polymorphism · Recombinant inbred lines

## Introduction

Hessian fly (HF), *Mayetiola destructor*, is an important pest of wheat worldwide. In the United States, the insect can be found in most wheat-growing regions (Ratcliffe and Hatchett 1997; Chen et al. 2009; Shukle et al. 2010). HF infestation in wheat can result in significant economic losses. The use of resistant cultivars is the most effective and economical approach for control of the pest (Berzonsky et al. 2003).

To date, 33 major HF resistance (HFR) genes have been identified from wheat and its relatives (Ratcliffe and Hatchett 1997; Harris et al. 2003; Martín-Sánchez et al. 2003; McIntosh et al. 2003; Williams et al. 2003; Liu et al. 2005a; Sardesai et al. 2005). Many of these resistance genes have been mapped to various wheat chromosomes across three genomes. Gallun and Patterson (1977) first mapped *H6* gene to chromosome 5A using monosomic analysis. Subsequently, other studies showed that *H3*, *H9*, and *H10* were all linked to *H6* (Carlson et al. 1978; Stebbins et al. 1982; Ohm et al. 1995). Further researches revealed that *H3*, *H5*, *H6*, *H9*, *H10*, *H11*, *H12*, *H14*, *H15*, *H16*, *H17*, *H19*, *H28*, *H29* and *Hdic* were all in the distal gene-rich region of wheat chromosome 1AS (Liu et al. 2005, Kong et al. 2005 & 2008), and formed an HFR-gene cluster (about 1 cM) close to markers *Xbarc263* and *Xcfa2153* (Liu et al. 2005). The majority of these HFR genes were derived from *T. turgidum* ssp. *durum*, except that *H3*, *H5*, and *H12* were from common wheat, and *Hdic* was from a cultivated emmer wheat (*Triticum turgidum* L. subsp. *dicoccon* (Schrunk) Thell.). Only *H20* (2B, Amri et al. 1990) and *H31* (5B, Williams et al. 2003) were mapped in the B genome of wheat, and *H13*, *H22*, *H23*, *H24*, *H26*, and *H32* were mapped on D genome. All HFR genes from the D genome were derived from *Ae. tauschii*, the D genome donor of common wheat (Martin et al. 1982; Gill et al. 1986, 1991a, b; Raupp et al. 1993; Cox and Hatchett 1994; Ratcliffe and Hatchett 1997; Sardesai et al. 2005), and located on chromosomes 1D,

3D, 4D, and 6D (Gill et al. 1987; Raupp et al. 1993; Cox and Hatchett 1994; Martín-Sánchez et al. 2003; Liu et al. 2005; Sardesai et al. 2005; Wang et al. 2006; Zhao et al. 2006; Yu et al. 2009; Yu et al. 2010). In addition to these HFR genes identified from wheat, *H21* and *H25* were derived from rye (*Secale cereale*) and transferred to common wheat (Friebe et al. 1996). Most of the wheat germplasm containing HFR genes have been used as parents in many U.S. breeding programs except *H21* which only became available recently after the rye chromosome fragment harboring *H21* was shortened (Cainong et al., 2010). However, due to lack of breeder-friendly diagnostic markers for most of these HFR genes, it is unknown how many have been actually deployed in commercially growing cultivars.

Many different HF biotypes have been identified based on their differential reactions to different R genes. Based on their virulence to *H3*, *H5*, and *H6* and a combination of *H7H8* (Ratcliffe et al. 1994, 1996, 1997, 2000), HF populations are classified into 16 biotypes designated as biotypes *A* to *O* and the Great Plains biotype (*GP*). Because the wheat and HF interaction is a gene-for-gene system, continuous evolution of new virulent biotypes in response to selection pressure from the HFR genes deployed in wheat cultivars can quickly overcome the single-gene resistance in a cultivar (Ratcliffe and Hatchett 1997, Gould 1998). Therefore, pyramiding several HFR genes against different biotypes may extend the life span of resistant cultivars. Molecular markers closely linked to these genes are essential for such gene pyramiding, however, many earlier reported genes were located to chromosomes using monosomic analysis (Gallun and Patterson 1977; Ohm et al. 1995). Some were mapped using DNA markers, but the mapping populations used were mainly F<sub>2</sub> generations (Williams et al. 2003; Liu et al. 2005a; Liu et al. 2005c; Wang et al. 2006; Kong et al. 2008; Yu et al. 2009; Miranda et al. 2010). Because only a single plant was phenotyped without replication, escape of infestation may cause significant

errors in phenotypic data. Thus, recombinant inbred populations provide more accurate phenotypic data by testing multiple plants per line to minimize errors due to infestation escape.

Classic gene mapping treats phenotypic data as binary data, the same as the DNA markers. This method is useful for single-gene mapping, but some resistant germplasm may have more than one gene, and may contribute partial resistance with an additive effect. In this case, classic linkage mapping may not be able to locate all the genes, so QTL mapping may provide a better way to locate all the genes in chromosomes and determine their individual effects.

To date, most of HFR genes have been identified from wheat relatives and are located in the 1AS cluster, thus identification of new HFR genes and associated markers from other wheat chromosomes will facilitate pyramiding of different HFR genes in breeding. Although HF biotype *GP* is the least virulent biotype and is only virulent to *H32* (Sardesai et al. 2005), it is still the predominant biotype in field populations. Therefore, identification of new HFR genes that are resistant to biotype *GP* is still useful for the pest management, especially when new HFR genes are located in different wheat chromosomes that can be used in gene pyramiding. ‘Clark’ is resistant to biotype *GP*, and mapping of R gene(s) in ‘Clark’ has not been reported. The objectives of this research were to 1) determine how many genes are involved in HFR in ‘Clark’, 2) identify the chromosome locations of these HFR genes, and 3) develop high-throughput molecular markers closely linked to these genes for MAS.

## **Materials and Methods**

### **Plant materials and evaluation of resistance to Hessian fly**

A population of 127 F<sub>12</sub> RILs was developed from the cross ‘Ning7840’ x ‘Clark’ by single-seed descent. ‘Clark’ is a soft red winter wheat cultivar derived from ‘Beau’ // ‘Caldwell’ sib / 67137B5-16 /4/ ‘Sullivan’ /3/ ‘Beau’ // 5517B8-5-3 -3 / Logan at Purdue University, West

Lafayette, IN (Ohm et al. 1988). It showed resistance to HF biotype *GP* and was thought to carry the *H6* gene (Ratcliffe et al. 2000). ‘Ning7840’ is a Chinese hard red facultative cultivar with the pedigree of ‘Aurora’ / ‘Anhui 11’ // ‘Sumai 3’, and is susceptible to HF biotype *GP*. The mapping population, two parents and four controls, Ike (*H3*), ‘Caldwell’ (*H6*), *H13*, and ‘Karl 92’ (susceptible control), were evaluated for reactions to infestation by HF biotype *GP* in fall 2011 and spring 2012, respectively, at Kansas State University, Manhattan, KS using a randomized complete block design. In each experiment, 20 seeds of each wheat cultivar or RILs were planted in uniformly spaced rows (24 half-rows per flat) in flats (52×36×10 cm) containing a mixture (1:1) of soil and vermiculite in growth chambers at  $18 \pm 1$  °C with 14:10 h (light:dark) photoperiod. Seedlings at the one-leaf stage were infested by confining ~200 newly mated HF females in each flat within a cheesecloth tent. Three weeks after infestation, all the seedlings from each RIL were examined to determine susceptible and resistant phenotypes. Susceptible plants were stunted with dark green leaves and harbored live larvae, whereas resistant plants grew normally with light green leaves and had dead larvae between the leaf sheaths. When otherwise normal plants contained some live larvae of much smaller sizes than in susceptible plants, the plants were still considered as resistant. Percentage of susceptible plants in a RIL was used for QTL analysis.

#### DNA extraction and marker analysis

Leaf tissue from five plants per line was sampled at the two-leaf stage in 1.1-mL deep-well plates and freeze-dried for 2 days (Thermo Fisher, Waltham, MA) for DNA isolation. Each well of the plates contained a 3.2-mm stainless steel bead and dried tissue, and the plates were shaken in a Mixer Mill (Retsch GmbH, Germany) at  $25 \text{ times s}^{-1}$  for 5 min. Genomic DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Saghai-Maroo et al., 1984). Polymerase chain reactions (PCR) were performed in a Tetrad Peltier DNA Engine (Bio-Rad Lab, Hercules,

CA) with a 12- $\mu$ L PCR mixture containing 1.2  $\mu$ L 10 $\times$  PCR buffer (Bioline, Taunton, MA), 2.5 mM  $MgCl_2$ , 200  $\mu$ M of each dNTP, 200 nM M13 fluorescent-dye-labeled primer (ACGACGTTGTAAAACGAC), 50 nM tailed forward primer (adding the M13 tail sequence to 5'-end of forward primer), 250 nM reverse primer, 0.6 U *Taq* DNA polymerase, and about 80 ng of template DNA. A touchdown PCR program was used for PCR amplification. Briefly, the reaction was incubated at 95°C for 5 min, then continued for five cycles of 1 min at 96°C, 5 min at 68°C with a decrease of 2°C in each subsequent cycle, and 1 min at 72°C. For another five cycles, the annealing temperature started at 58°C for 2 min, with a decrease of 2°C for each subsequent cycle. Reactions then went through an additional 25 cycles of 1 min at 96°C, 1 min at 50°C, and 1 min at 72°C with a final extension at 72°C for 5 min. PCR products were separated on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Data collected from an ABI DNA Analyzer (Applied Biosystems) were processed by GeneMarker version 1.6 (Soft Genetics LLC, State College, PA) and manually checked twice for accuracy.

SNP genotyping was performed on the BeadChip array platform containing 9000 wheat SNP markers using the Infinium<sup>TM</sup> iSelect SNP genotyping assays developed by Illumina Inc. (San Diego, CA). The assay was designed under the protocols of the International Wheat SNP Consortium (Cavanagh et al. 2013). SNP genotype calling was performed using GenomeStudio v2011.1 software (Illumina, San Diego CA). The genotyping assay was conducted at the USDA Small Grains Genotyping Lab in Fargo, ND.

### Linkage map construction and QTL determination

The linkage map was constructed using the MAP function in software QTL IciMapping 3.2 (Wang et al. 2012) with a minimum LOD value of 5.0. Map distance used the Kosambi mapping function. The ordering of markers and assignment of linkage groups to chromosomes referred to a



previously published wheat consensus map (Somers et al. 2004).

QTLs were mapped with QTL IciMapping version 3.2 using inclusive composite interval mapping of additive (ICIM-ADD) and epistatic QTL (ICIM-EPI) modules. Additive QTL was detected using a 1.0 cM step in scanning. The probability used in stepwise regression was 0.001. Significant LOD thresholds were determined for each dataset by 1000 permutations. Type I error rate to determine the LOD threshold from permutation tests was 0.05. Epistatic QTL were detected using a step of 5 cM in scanning, probability of 0.0001 in stepwise regression, and a LOD threshold of 5.0 to claim the significant QTL.

## Results

### Phenotypic reactions to HF biotype *GP* infestation

All plants of ‘Ning7840’ and susceptible-control ‘Karl 92’ were susceptible to HF biotype *GP* infestation, whereas all plants of ‘Clark’ and resistant-control ‘Caldwell’ (*H6*), ‘Molly’ (*H13*) were resistant to biotype *GP*. Cultivar ‘Ike’ showed heterogeneous phenotypes, with most plants showing a resistant reaction. The mapping population segregated with 82 homozygous resistant RILs, 38 homozygous susceptible RILs, and 6 heterogeneous RILs in the winter 2011 test, and with 68 homozygous resistant, 36 homozygous susceptible, and 23 heterogeneous RILs in the spring 2012 test. The segregation ratio of resistant and susceptible RILs deviated from 1:1, a single gene segregation ratio, suggesting that at least two genes were involved in resistance to HF in ‘Clark’. Quantitative variation in resistance to HF biotype *GP* was observed in some RILs. In some resistant plants, seedlings grew normally without any injury to plant tissue at feeding sites and the larvae were dead within 2 to 3 days after infestation, but in other resistant plants larvae were alive for a longer time period (up to 5 days) and the size of dead larvae became bigger. In both cases, the

dead larvae remained reddish (color of the first instar), indicating that the larvae in the latter case might grow more, but are unable to develop into second instar. Most susceptible plants showed stunting and dark green coloration with large living larvae between leaf sheaths, whereas some seedlings looked relatively normal in appearance (with some growth) and had small living larvae in leaf sheaths. These quantitative phenotypic variations also suggest that more than one gene controls the resistance to HF.

### Linkage map and QTLs for HFR

The RIL population was analyzed with 593 SNPs and 218 SSRs polymorphic between the two parents. A total of 805 markers (99%) were assigned to 42 linkage groups representing all 21 wheat chromosomes and covering a total distance of 3728.3 cM with an average interval length of 4.6 cM.

Using the ICIM mapping program, two QTLs associated with HF resistance were identified in both 2011 and 2012 experiments and in the mean over the two experiments (Table 1). These were located on chromosomes 6B and 1A with the ‘Clark’ alleles increasing HFR. The major QTL on 6B was positioned between markers *Xsnp2745* and *Xsnp921* at 4.5 cM apart (Fig. 1). Eight additional SNPs were mapped in the QTL region. This QTL explained 37.8% and 41.6% of the phenotypic variation with LODs of 14.2 and 16.1 in the 2011 and 2012 experiments, respectively, and 37.2% of the phenotypic variation for the mean over the two experiments with a LOD of 16.6. The second QTL on chromosome 1A accounted for 10.8% and 10.3% of the phenotypic variation in the two experiments and 13.3% of the phenotypic variation for the mean with LOD values of 4.5 (2011), 4.6 (2012), and 6.5 (mean over two experiments). This QTL was located in the marker interval *Xgwm33-Xsnp5150* spanning about 6.0 cM. Four additional markers were mapped in the region. Two QTLs together explained 54.7% the phenotypic variation for the mean over the two

experiments (Table 1).

### Epistatic QTL for HFR

Using epistatic QTL (ICIM-EPI) modules, one pair of epistatic QTL that located on chromosome 6B and 1A was observed both in 2011 and 2012 experiments, and also in the mean over the two experiments (Table 2). This epistatic QTL was positioned in the marker interval *Xsnp5780-Xsnp921* on 6B over the two experiments coinciding with the 6B main effect QTL, and *Xsnp5150-Xsnp4754* on 1A near the 1A main effect QTL. It explained additional 22.0%, 18.5% of the phenotypic variations for HFR with LOD 20.8 and 20.0 in 2011 and 2012 experiments, respectively, and 9.5% of the phenotypic variation for the mean over two experiments with LOD of 5.7.

### Discussion

In this study, we used a RIL population instead of  $F_2$  as reported in most previous studies (Dweikat et al. 2002; Martín-Sánchez et al. 2003; Kong et al. 2005, 2008; Liu et al. 2005b, 2005c; Yu et al. 2009) to improve phenotyping accuracy. RILs have a high recombination frequency resulting from multiple meiotic events that occurred during repeated selfing (Jansen 2003), and a high level of homozygosity that enables replicated phenotyping across different environments. In this study,  $F_{10-12}$  RILs were evaluated for HFR, and some RILs showed homogeneous phenotypes in one experiment, but heterogeneous phenotypes in the other. Most of these RILs should be homozygous genotypes. The same heterogeneous phenotypes were observed for check 'Ike'. This result suggests that HFR evaluation based on a single plant may not be accurate, thus, phenotyping multiple plants per genotype in repeated experiments can significantly improve the accuracy of phenotypic data for HFR gene mapping. In this study, the same RILs were used for repeated phenotyping. The RILs were evaluated for HFR using a large number of plants (20 plants) per RIL,

and phenotypic data were scored as the percentage of resistant plants in each RIL tested. Although phenotypic variation was observed for some individual RILs between experiments, QTL were mapped unequivocally in the same regions of 6B and 1A using data from both experiments. In addition, a high-density map has never been used for HFR gene mapping. Resolution of all previous maps was usually poor, so closely linked markers were not identified. In this study, a high-resolution map with 805 markers was used for mapping QTLs for HFR, which provides greater precision for QTL location detected and better marker coverage in the QTL region than previous study.

Using the new map, we identified two QTLs on 1A and 6B in both experiments using a high-density map of 805 markers. One HFR QTL was detected on the chromosome 1A of ‘Clark’, designated as *Qhf-hwwg-1A*, and very closely linked to *Xgwm33*, a marker closely linked to 1A gene cluster of 15 HFR genes (Stebbins et al. 1983; Roberts and Gallun 1984; Liu et al. 2005a, 2005b; Kong et al. 2005, 2008). This QTL is likely *H6* derived from ‘Caldwell’ (Ratcliffe et al., 2000; Chen et al. 2009) and appears to contribute a minor effect (accounting for about 10% of the phenotypic variance) to resistance to *GP* biotype in this study. To date, many molecular markers, including SSR, randomly amplified polymorphic DNA, and sequence tag sites, for HFR genes in 1AS cluster have been published (Dweikat et al. 1997, 2002; Liu et al. 2005a, 2005b; Kong et al. 2005, 2008; Bouktila et al. 2006). SNP markers that are suitable for high-throughput screening have not been reported in the 1AS cluster. In this study, we identified two flanking markers, *Xgwm33* and *Xsnp5150*, and four additional SNPs, *Xsnp4505*, *Xsnp4351*, *Xsnp1970*, and *Xsnp6649*, in the *Qhf-hwwg-1A* QTL region. These markers should be useful for MAS of the QTL identified in this study, and for other genes in the cluster after further validation in different genetic backgrounds.

Another QTL with a major effect on HFR was detected on the 6BS of ‘Clark’. To date, no gene has been reported from chromosome 6B among the 33 known HFR genes. This is most likely a novel HFR gene in wheat, designated *H34*, located distally to *Xwmc494*, and flanked by two SNP markers, *Xsnp921* and *Xsnp2475*. Eight additional SNP markers, *Xsnp2479*, *Xsnp6760*, *Xsnp6759*, *Xsnp2477*, *Xsnp6704*, *Xsnp1494*, *Xsnp1495*, *Xsnp2476*, were also mapped in the QTL region. Those markers are very close to *H34* and should be useful for marker-assisted pyramiding of this gene with those genes from other chromosomes to improve wheat for HFR.

Besides the main additive effect of the two QTLs, we also detected a stable epistasis between the two QTLs across all the experiments. This epistatic QTL were positioned in the *H34* region and near the *Qhf-hwwg-1A*, and accounted for additional 9.5% of the mean phenotypic variation and decreased HF score by 0.16, which showed that the epistasis had a positive effect on HFR. The epistatic QTL on 1A is about 6 cM from main effect QTL *Qhf-hwwg-1A*, so it is likely the same QTL with both main and epistatic effects. The small difference in the position between QTLs for main effect and for epistasis was possibly due to phenotyping error. The results from this study indicate that HFR genes may contribute quantitative resistance to HF infestation. Different genes may contribute different degrees of resistance in a cultivar, and epistasis may play significant roles in control of HFR.

### **Acknowledgment**

This is contribution number 13-002-J from the Kansas Agricultural Experiment Station. This project is partly funded by the National Research Initiative Competitive Grants CAP project 2011-68002-30029 from the USDA National Institute of Food and Agriculture. Authors also thank International Wheat SNP Consortium for assembling wheat 9K iSelect chips. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific

information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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Table 1 Chromosome (Chr.) locations, peak positions (cM), marker intervals, LOD values, phenotypic variations explained (PVE), additive effects of quantitative trait loci (QTLs) detected for Hessian fly resistance using ‘Ning7840’/‘Clark’ recombinant inbred population

QTL	Chr.	Peak position	Marker interval	2011			2012			Mean		
		(cM)		LOD <sup>a</sup>	PVE <sup>b</sup>	ADD <sup>c</sup>	LOD <sup>a</sup>	PVE <sup>b</sup>	ADD <sup>c</sup>	LOD <sup>a</sup>	PVE <sup>b</sup>	ADD <sup>c</sup>
<i>H34</i>	6B	49	<i>Xsnp247-Xsnp921</i>	14.2	37.8%	-0.28	16.1	41.6%	-0.28	16.6	37.2%	-0.27
<i>QHf-hw</i> <i>wg-1A</i>	1A	4	<i>Xsnp515-Xgwm33</i>	4.5	10.8%	-0.15	4.6	10.3%	-0.14	6.5	13.3%	-0.16
Total					46.4%			49.9%			54.7%	

<sup>a</sup> LOD peak value at the center of the QTL.

<sup>b</sup> Phenotypic variation explained by the QTL.

<sup>c</sup> Additive effect. A negative additive effect value implies that the ‘Clark’ allele increase the resistance to HF.

Table 2 Chromosome (Chr.) location, flanking markers, LOD value, phenotypic variation explained (PVE) by epistatic QTL, and epistatic effect (AA) of epistatic QTL for HFR identified on chromosomes 6B and 1A.

Experiment	Chr.1	Site1 (cM)	Flanking markers	Chr.2	Site2 (cM)	Flanking markers	LOD <sup>a</sup>	PVE <sup>b</sup> (%)	AA <sup>c</sup>
2011	6B	49	<i>Xsnp2475-Xsnp921</i>	1A	10	<i>Xsnp5150-Xsnp4754</i>	20.8	22.0	-0.22
2012	6B	44	<i>Xsnp5780-Xsnp2475</i>	1A	10	<i>Xsnp5150-Xsnp4754</i>	20.0	18.5	-0.18
Mean	6B	49	<i>Xsnp2475-Xsnp921</i>	1A	10	<i>Xsnp5150-Xsnp4754</i>	5.7	9.5	-0.16

<sup>a</sup> LOD score for epistatic QTL.

<sup>b</sup> Phenotypic variation explained by the epistatic QTL effects.

<sup>c</sup> Additive by additive effect of QTL at the two scanning positions. A negative additive effect value implies that an interaction between two epistatic ‘Clark’ alleles increases the resistance to HF.

Fig. 1 Two quantitative trait loci for Hessian fly resistance were detected on chromosomes 6B (left) and 1A (right) in the ‘Ning7840’ / ‘Clark’ mapping population. Y-axis of QTL map is LOD value and X-axis is map distance as indicated in the linkage maps. Dashed line parallel to the X-axis is the LOD threshold for significant QTL derived from permutation tests. *H34* was positioned between markers *Xsnp921* and *Xsnp2745*; *QHf-hwwg-1A* was flanked by markers *Xgwm33* and *Xsnp5150*.

